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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		**Docket No.:**	**4240-116**
Applicants:	**SUNG, Moon-Hee, et al.**	**Conf. No.:**	**5278**
Application No.:	**10/520,557**	**Art Unit:**	**1609**
Date Filed:	**January 6, 2005**	**Examiner:**	**Susanna Hoffer**
Title:	**POLY-GAMMA-GLUTAMATE HAVING ULTRA HIGH MOLECULAR WEIGHT AND METHOD FOR USING THE SAME**	**Customer No.:**	**23448**

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Kristin Robinett

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April 28, 2008

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SUBMISSION OF CERTIFIED COPY OF PRIORITY KOREAN PATENT APPLICATION NUMBER 10-2002-0040083, TO PERFECT PRIORITY CLAIM IN U.S. PATENT APPLICATION NO. 10/520,557

Commissioner for Patents
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Alexandria, Virginia 22313-1450

Sir:

Enclosed is a certified copy of priority Korean Patent Application No.10-2002-0040083, to perfect the priority claim in the above-identified U.S. patent application.

Respectfully submitted,

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Enclosures:
Certified Copy of Priority Korean Patent Application Number 10-2002-0040083 [18 pgs.]
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[SPECIFICATION]

[NAME OF INVENTION]

POLY-GAMMA-GLUTAMATE WITH VERY HIGH MOLECULAR WEIGHT AND METHOD FOR USING THEREOF

[BRIEF DEACRIPTION OF DRAWINGS]

FIG. 1 is a graph showing the molecular weight distribution of the PGA according to the present invention;

FIG. 2 is a graph showing the comparison between the water-absorbing property of the ultra-high molecular weight PGA of the present invention and a product of the prior art;

FIG. 3 is a graph showing the comparison between the moisture-retaining property of the ultra-high molecular weight PGA of the present invention and a product of the prior art;

FIG. 4 is a graph showing an effect of the ultra-high molecular weight PGA according to the present invention on the improvement of Ca solubility;

FIG. 5 shows an effect on water absorption of a hydrogel produced from the ultra-high molecular weight PGA of the present invention.

[DETAILED DISCLOSURE OF INVENTION]

[OBJECT OF INVENTION]

[TECHNICAL FIELD AND PRIOR ART]

The present invention relates to an ultra-high molecular weight poly-gamma-glutamate (hereinafter, referred to as "PGA") produced by a

halotolerantstrain *Bacillus subtilis var. chungkookjang* (KTCT 0697BP) isolated from chungkookjang, Korean traditional fermented soybean food, and also to the method of use thereof. More particularly, the present invention relates to a PGA with a molecular weight greater than 10,000 kDa showing edibility, water solubility, an anionic property and biodegradability, and also to foods, cosmetics, feedstuffs, mineral absorption-promoting compositions, which contain the same.

PGA is a viscous polymer where D,L-glutamate is polymerized through gamma-glutamyl. It is produced from a *Bacillus* sp. strain, which is isolated from chungkookjang as Korean traditional food obtained from the fermentation of soybeans using rice-straw, natto as Japanese traditional fermented soybean food, and kinema as Nepalese traditional fermented soybean food.

The PGA produced from the *Bacillus* sp. strain is a polymer having edibility, water solubility, an anionic property and biodegradability, and can be used as a raw material of moisture-absorbing agents, moisture-retaining agents and cosmetics, and a raw material for the preparation of naturally degradable plastics using the synthesis of ester derivatives.

Recently, with respect to the production and use of the PGA, there are being actively conducted studies on the development of a material as a substitute for difficultly degradable polymers, and the production of heat-resistant plastics by esterication, and the production of water-soluble fibers and membranes, etc., in highly developed countries as a leader. Furthermore, studies on a change in physical properties of the PGA occurring upon irradiation of the PGA with gamma rays, and studies on the development and industrial application of a PGA hydrogel using crosslinkers.

The PGA hydrogel is an environment-friendly material, which is produced by the intermolecular or intramolecular crosslinking of the PGA, a biopolymer produced by the culturing of *Bacillus subtilis var. chungkookjang,*

and has a water-absorbing property, biodegradability and thermoplasticity. Methods for the crosslinking of the PGA include irradiation with radiation, such as gamma rays or electron beams, treatment with chemical crosslinkers, such as epoxy resin, and the like. When aqueous PGA solution is irradiated with radiation, the crosslinking between PGA molecules takes place, thereby giving PGA resin having a water-absorbing property, biodegradability and thermoplasticity.

In the prior art, there were reported a study on an effect of manganese ions on the composition and production of PGA, a study on the production of the PGA having water solubility by ultrasonic decomposition, and a study on the production of plastics of low water solubility by synthesis with ester derivatives (*Biosci. Biotechnol. Biochem.*, 60(8):1239-42, 1996), a study on the production of PGA using *Bacillus subtilis,* and a study on the use of the PGA for healthy foods having a therapeutic effect of osteoporosis, such as a calcium-dissolving agent, etc. (Japanese patent laid-open publication No. Heisei 6-32742).

In addition, there was reported an effect of PGA on the reduction of water contamination according to the reduction of a phosphorus content in a water system (European patent No. 838160). Moreover, highly gelling, water-soluble, biodegradable and adsorbent PGA resins, and the use thereof for sanitary products and foods and in horticultural industries, etc., were disclosed (Japanese patent laid-open publication Nos. Heisei 10-251402, 7-300522 and 6-322358).

Furthermore, there were known the use of PGA for solid biodegradable fibers, films or film-shaped materials by the dissolution, precipitation and drying of the PGA (Japanese patent laid-open publication Nos. Heisei 7-138364 and 5-117388), and the use of the PGA for a drug carrier (Japanese patent laid-open publication Nos. Heisei 6-92870 and 6-256220).

Meanwhile, there were known inventions on the efficient production of

the PGA (Korean patent application No. 1997-3404, Korean patent application No. 1997-67605), the production of high concentration PGA (Korean patent application No. 2001-0106025), and halotolerantstrain *Bacillus subtilis var. chungkookjang* of producing a high-molecular weight PGA (PCT application No. PCT/KR01/01372 corresponding to Korean patent laid-open publication No. 2001-01481).

The molecular weight of PGAs produced in the prior art is in the range of about 100-2,000 kDa, and they have limitations on the application thereof, particularly in cosmetic or food fields, in terms of the solubility, absorption and sustained release of minerals.

Accordingly, the present inventors have conducted extensive studies in an attempt to produce an ultra-high molecular weight PGA, and consequently, found that the batch culturing of *Bacillus subtilis var. chungkookjang* in medium containing glucose, citric acid and glutamate yielded a PGA having a molecular weight greater than 10,000 kDa without byproducts, and the produced PGA showed a very excellent effect upon the use thereof for moisture-retaining agents, water-absorbing agents, and mineral absorption-promoting agents. On the basis of this point, the present invention was perfected.

Therefore, a main object of the present invention is to provide a PGA having an ultra-high molecular weight greater than 10,000 kDa.

[CONSTITUTION OF INVENTION]

To achieve the objects as described above, the present invention provides an ultra-high molecular weight PGA having a mean molecular weight greater than 3,000 kDa.

Preferably, the molecular weight of the PGA according to the present invention is in more than 6,000 kDa, more preferably 10,000 kDa.

Since the PGA according to the present invention has ultra-high molecular weight, it has very excellent moisture-absorbing and moisture-retaining properties as compared to the prior PGA with relatively low molecular weight.

The PGA according to the present invention has a very excellent property of enhancing the solubility of Ca ions, and an excellent property on the sustained release of mineral ions. Thus, the present invention relates to a method for applying to improve Ca solubility with the ultra-high molecular weight PGA.

A hydrogel produced from the PGA of the present invention as a raw material has a very excellent water-absorbing property as compared to the prior product with relatively low molecular weight. Thus, the present invention also provides a hydrogel produced from the ultra-high molecular weight PGA, as well as a moisture-absorbing or water-absorbing agent containing the same.

Furthermore, the present invention provides a method for using the ultra-high molecular weight PGA with a molecular weight greater than 5,000 kDa, for a mineral absorption-promoting agent.

In the present invention, the ultra-high molecular weight PGA is produced by microbial culturing. A microorganism used for the production of the ultra-high molecular weight PGA in the present invention is *Bacillus subtilis var. chungkookjang* (KCTC 0697BP) whose isolation, identification and physiological characteristics are described in detail in PCT application No. PCT/KR01/01372, which was filed in the name of the present inventors on August 11, 2001.

The morphological and physiological characteristics of this strain are as follows.

This strain is gram-positive bacteria, which form milky colonies upon culturing on an LB agar plate, and show active growth in aerobic conditions

above 37 °C and slow growth at a culturing temperature higher than 55 °C. Furthermore, this strain is a halotolerant strain that can grow even at a salt (NaCl) concentration of 9.0%, which is higher than the salt tolerance of general *Bacillus subtilis* species. Also, it is a typical *Bacillus* strain, which forms endospores when it is cultured in LB liquid medium or solid medium for at least 70 hours. The comparative analysis of the 16S rDNA sequence of this strain and the 16S rDNA sequence of the prior *Bacillus* sp. strain reveals that this strain has a very high homology of 99.0% with *Bacillus subtilis*.

In the present invention, a mean molecular weight of PGA produced by a halotolerantstrain *Bacillus subtilis var. chungkookjang* (KTCT 0697BP) is 13,000 kDa, and more than 95% thereof are in the range of 3,000 ~15,000kDa. Therefore, if necessary, the PGA can be cut by a proper method to obtain PGAs having a certain molecular weight, or can be separated to collect as certain molecular weight to use.

The present invention will hereinafter be described in further detail by examples. It should however be borne in mind that these examples are given for illustrative purpose only and the scope of the present invention is not limited to or by the examples.

Although the production of the ultra-high molecular weight PGA using *Bacillus subtilis var. chungkookjang* (KCTC 0697BP) was illustrated in the examples, it is to be understood that a PGA produced by other strains or chemical methods falls within the technical scope of the present invention as long as it is an ultra-high molecular weight PGA.

Example 1: Production and molecular weight measurement of ultra-high molecular weight PGA

A 5L fermenter containing 3L minimal medium (GS medium

containing 4% L-glutamate, 3% glucose, 1% $(NH_4)_2SO_4$, 1% Na-citrate, 0.27% KH_2PO_4, 0.42% Na_2HPO_4, 0.05% NaCl, 0.3% $MgSO_4$, 1 ml/L vitamin solution, pH 6.8) was inoculated with 1% of a culture broth of *Bacillus subtilis var. chungkookjang* (KCTC 0697BP), and cultured at a stirring speed of 150 rpm, an aeration rate of 1 vvm, and 37 °C for 3 days, and then adjusted to pH 3.0 by the addition of 2N sulfuric acid solution, thereby obtaining a PGA-containing sample solution.

The sample solution was left to stand at 4 °C for 10 hours to remove polysaccharides present in the fermented solution, and added with ethanol at the amount of two times volume larger than the fermented solution, and then mixed thoroughly. The mixed solution was left to stand at 4 °C for 10 hours, followed by centrifugation, to give a PGA precipitate.

The precipitate was dissolved by the addition of distilled water, added with 100 μg/ml protease, and allowed to react in a 37 °C incubator for 6 hrs, thereby decomposing extracellular protein present in the PGA sample.

The resulting substance was dialyzed against a sufficient amount of distilled water to remove free glutamate, followed by concentration, to give pure PGA.

As shown in FIG. 1, it could be found by GPC analysis that the mean molecular weight of the PGA obtained as described above is 13,000 kDa, and more than 95% of its molecules have a molecular weight ranging from 3,000 to 15,000 kDa.

Example 2: Moisture-absorbing and moisture-retaining properties of ultra-high molecular weight PGA

The moisture-absorbing and moisture-retaining properties of the ultra-high molecular weight PGA produced in Example 1 were compared to an existing PGA having a molecular weight of 600 kDa.

(1) Comparison of moisture-absorbing property

0.5g of each of the PGA obtained in Example 1 and a prior product with a molecular weight of 600 kDa were put in the respective Petri dish and maintained in a 45 °C incubator for 14 hours to remove water completely. The resulting samples put in a decicator (relative humidity: 81-88%) containing a saturated aqueous solution of calcium carbonate (250g calcium carbonate per 500g purified water), and were measured for a change in its weight according to time (moisture-absorbing property) for 24 hours. The measured results are shown in FIG. 2.

As shown in FIG. 2, it was found that the PGA with a 600-kDa molecular weight showed less than 10% increase in water content after 24 hours, whereas the PGA according to the present invention showed about 60% increase in water content, indicating an extraordinarily excellent moisture-absorbing property of the inventive PGA.

(2) Comparison of moisture-retaining property

Samples, which had been sufficiently moisturized by standing for 48 hours under the conditions described in the above test (1), were put in a decicator (18% humidity) containing 500g dry silica gel and measured for a reduction in its water content according to time (moisture-retaining property) for 24 hours at 25 °C (FIG. 3).

As shown in FIG. 3, it was found that the prior PGA with a 600 kDa molecular weight showed 13% reduction in its water content after 24 hours, whereas the ultra-high molecular weight PGA of the present invention showed about 10% reduction in its water content, demonstrating a very excellent moisture-retaining property of the inventive PGA.

From the results of this example, it can be found that the ultra-high molecular weight PGA of the present invention can be used for a variety of

moisture-retaining and/or moisture-absorbing products.

Example 3: Ca solubility of the ultra-high molecular weight PGA

In order to examine the Ca solubility of the ultra-high molecular weight PGA of the present invention, the following test was carried out.

The ultra-high molecular weight PGA produced in Example 1 was diluted to prepare PGA solutions having concentrations of 0.062, 0.125, 0.25 and 0.5 mg/ml, respectively. 0.5 ml of each of the PGA solutions was added to a reaction solution containing 0.5 ml of 10mM $CaCl_2$ and 1.0 ml of 20 mM phosphate buffer, followed by reaction at 37 °C. After 2 hours, the respective solutions were centrifuged at 2000g for 30 minutes, and Ca remaining in the supernatant was quantified with a Ca quantification kit (Wako Chemical Co., Japan) (FIG. 4).

As shown in FIG. 4, the inventive PGA dissolved (adsorbed) Ca ions at a significantly larger amount than the prior products over all the concentrations. Particularly, at a PGA concentration of 0.5 mg/ml, the prior PGA with a 600 kDa molecular weight showed Ca solubility of about 40%, whereas the ultra-high molecular weight PGA showed a Ca solubility of about 80%, which is twice as much effect.

From the results of this example, it can be found that the ultra-high molecular weight PGA of the present invention can be used in industrial products or edible products for Ca-absorption.

Example 4: Water-absorbing property of ultra-high molecular weight PGA hydrogel

5% aqueous solution of each of the ultra-high molecular weight PGA produced in Example 1 and a prior PGA product (600 kDa) was irradiated with gamma ray of 25 kGy, thereby producing hydrogels.

Then, each of the produced hydrogels was immersed in water, and after

24 hours, measured for its weight in water, thereby examining a water-absorbing property of the hydrogels (FIG. 5).

As shown in FIG. 5, the prior PGA hydrogel absorbed 2000 times its weight in water, but the inventive PGA hydrogel absorbed 6400 times its weight in water, that indicates 3 times higher water absorption capability than that of the hydrogel containing the prior PGA product.

As a result, it can be found that water-absorbing hydrogel produced from the inventive PGA shows an excellent effect of absorbing an increased amount of water even at a lower volume than hydrogel produced from the prior PGA.

[EFFECT OF INVENTION]

As described above, the present invention provides the ultra-high molecular weight PGA having a molecular weight greater than 10,000 kDa. Furthermore, the present invention provides cosmetics, materials for prompting a Ca-absorption, and highly water-absorbable hydrogel; and applicable methods using them.

WHAT IS CLAIMED IS:

1. An ultra-high molecular weight poly-gamma-glutamate (PGA) having a mean molecular weight greater than 3,000 kDa.

2. The PGA according to claim 1, which has a mean molecular weight greater than 6,000 kDa.

3. The PGA according to claim 1, which has a mean molecular weight greater than 10,000 kDa

4. A method for using the PGA according to any one of claims 1 to 3 for displaying a moisture-absorbing property and a moisture-retaining property.

5. A method for using the PGA according to any one of claims 1 to 3 for improving Ca solubility.

6. A hydrogel containing the PGA according to any one of claims 1 to 3.

7. The PGA according to any one of claims 1 to 3, which is produced by *Bacillus subtilis var. chunkookjang* (KCTC 0697BP).

[ABSTRACT]

The present invention relates to a poly-gamma-glutamate (PGA) having an ultra-high molecular weight greater than 3,000 kDa.

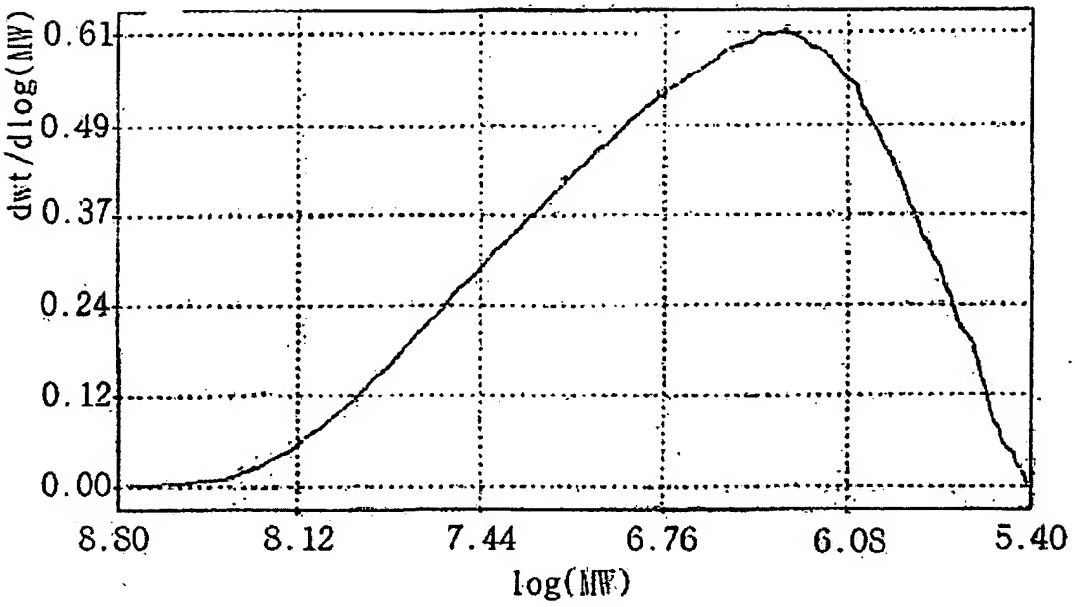
The ultra-high molecular weight PGA according to the present invention has a mean molecular weight greater than 13,000 kDa, and more than 95% of its molecules have a molecular weight ranging from 3,000 to 15,000 kDa. Also, it can be produced by the culturing of *Bacillus subtilis var. chungkookjang*.

The ultra-high molecular weight PGA according to the present invention shows very excellent moisture-absorbing, moisture-retaining, Ca solubility, and water-absorbing properties, and thus, can be used as a new and high value-added material in various applications.

Representative Figure : FIG.1

Guide word : poly-gamma-glutamate, ultra-high molecular weight, moisture-absorbing property, moisture-retaining property, hydrogel

FIG. 1


dwt/dlog(MW)
0.61
0.49
0.37
0.24
0.12
0.00
8.80
8.12
7.44
6.76
6.08
5.40
log(MW)

5

FIG. 2

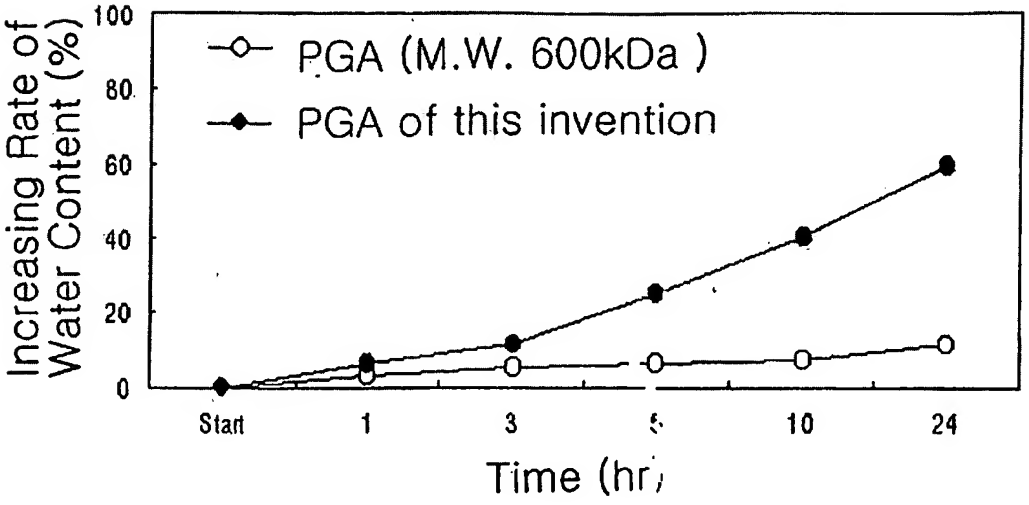

Increasing Rate of Water Content (%)
100
80
60
40
20
0
PGA (M.W. 600kDa)
PGA of this invention
Start
1
3
5
10
24
Time (hr)

FIG. 3

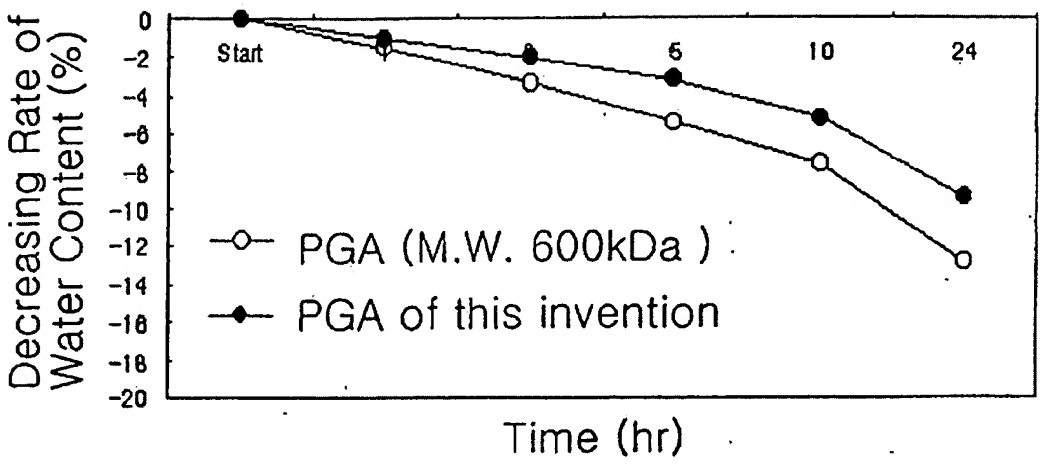

Decreasing Rate of Water Content (%)
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
Start
5
10
24
PGA (M.W. 600kDa)
PGA of this invention
Time (hr)

FIG. 4

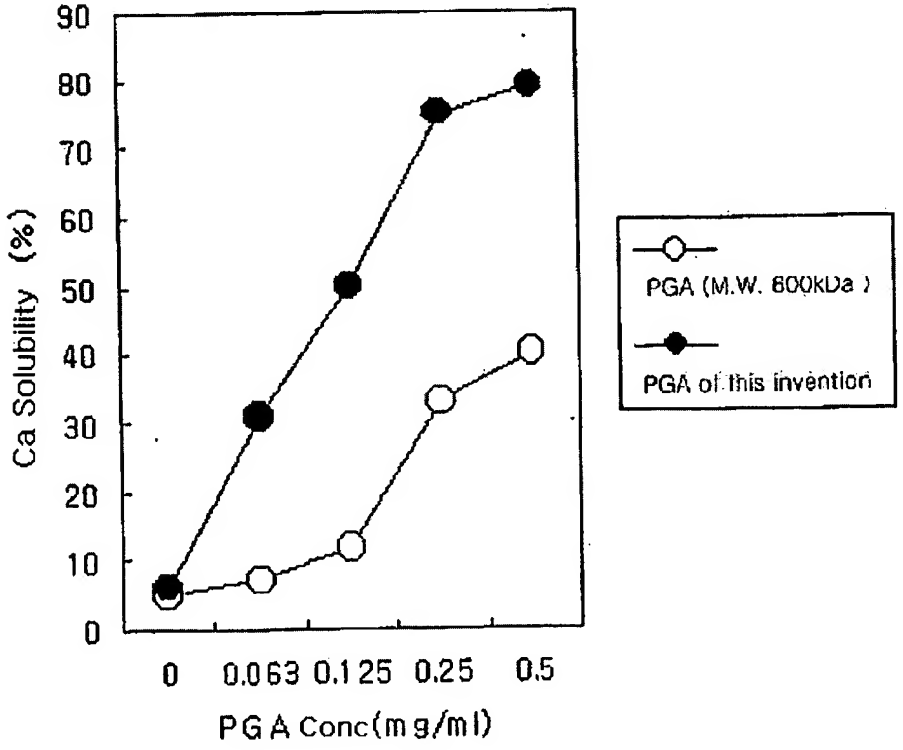
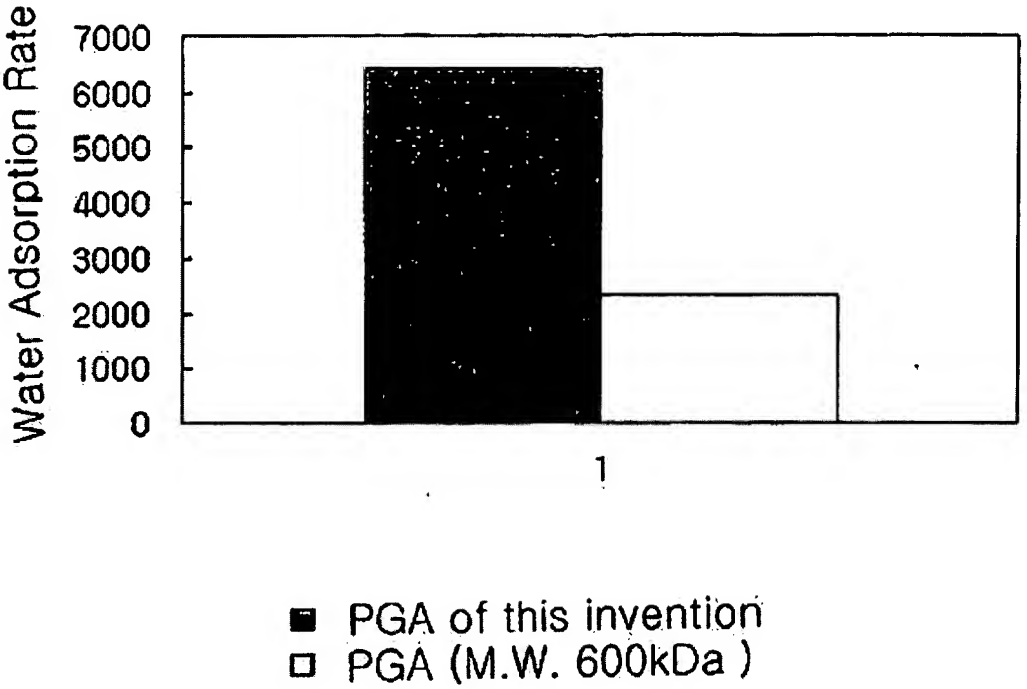

Ca Solubility (%)
90
80
70
60
50
40
30
20
10
0
0
0.063
0.125
0.25
0.5
PGA Conc(mg/ml)
PGA (M.W. 600kDa)
PGA of this invention

FIG. 5


Water Adsorption Rate
7000
6000
5000
4000
3000
2000
1000
0
1
PGA of this invention
PGA (M.W. 600kDa)

Amendment(2003.02.06)

[Subject] claim 1

[Method] amended

[Content]

An ultra-high molecular weight poly-gamma-glutamate (PGA) having a mean molecular weight greater than 5,000 kDa.

[Subject] claim 4

[Method] deleted

[Subject] claim 5

[Method] deleted

[Subject] claim 8

[Method] added

[Content]

Cosmetics containing the PGA according to any one of claims 1 to 3.

[Subject] claim 9

[Method] added

[Content]

Foods containing the PGA according to any one of claims 1 to 3.

[Subject] claim 10

[Method] added

[Content]

Medical supplies containing the PGA according to any one of claims 1 to 3.

[Subject] claim 11

[Method] added

[Content]

Feedstuffs containing the PGA according to any one of claims 1 to 3.

[Subject] claim 12

[Method] added

[Content]

A water-absorbing agent containing the PGA according to any one of claims 1 to 3.

[Subject] claim 12

[Method] added

[Content]

A supplement for Ca-absorption containing the PGA according to any one of claims 1 to 3.

Amendment(2003.05.28)

[Subject] claim 10

[Method] deleted

5

CERTIFICATE OF TRANSLATION OF KOREAN PATENT

APPLICATION NO. 10-2002-0040083

I, Lee, Cheo-young, hereby certify that the attached English language document entitled "POLY-GAMMA-GLUTAMATE WITH VERY HIGH MOLECULAR WEIGHT AND METHOD FOR USING THEREOF" is a true and accurate English translation of the text of Korean Patent Application No. 10-2002-0040083 as filed on July 10, 2002 in the Korean Intellectual Property Office and entitled "POLY-GAMMA-GLUTAMATE WITH VERY HIGH MOLECULAR WEIGHT AND METHOD FOR USING THEREOF."

(name of translator)

Date: 15 Apr. 2008